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Differential Sensitivity of Rat Kidney and Liver to Fumonisin Toxicity: Organ-Specific Differences in Toxin Accumulation and Sphingoid Base Metabolism

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Fumonisins (FBs) are mycotoxins in maize and are inhibitors of ceramide synthase (CS), the most likely proximate cause of FB toxicity. In liver and kidney, the primary target organs in FB-fed rats, inhibition of CS results in a marked increase in the ceramide precursor sphinganine (Sa). This study was conducted to investigate the differential time- and dose-dependent changes in Sa, sphingosine (So), sphinganine 1-phosphate (Sa-1-P), and sphingosine 1-phosphate (So-1-P) in kidney, liver, serum, and heart of male Sprague-Dawley rats (3–4 weeks old) fed diets containing 1.1, 13.5, and 88.6 μg/g of total FB for 10 days. The tissues were microscopically examined for the presence and severity of lesions consistent with FB exposure. There was a time- and dose-dependent increase in Sa in both liver and kidney, which was closely correlated with the tissue concentration of fumonisin B_1 (FB_1) and histopathologic findings. However, the Sa alone greatly underestimated the degree of disruption of sphingolipid metabolism since accumulated Sa and So were quickly metabolized to Sa-1-P and So-1-P as evidenced by large increases in these metabolites in kidney but not in liver. The concentration of FB_1 in liver and kidney that first elicited an increase in Sa was similar in both tissues, however, over time, the kidney accumulated significantly more FB_1 (10×) and total Sa (Sa plus Sa-1-P) compared to liver. Thus, the relative sensitivity of male Sprague-Dawley rat kidney and liver is most likely a consequence of differences in the mechanisms responsible for both FB_1 uptake/clearance and Sa metabolism.

Key Words: **fumonisin; sphingolipids; sphinganine; sphinganine 1-phosphate; sphingosine 1-phosphate.**

Fumonisins (FB) are carcinogenic mycotoxins (IARC, 2002) that commonly contaminate maize worldwide (Marasas, 2001). They are inhibitors of ceramide synthase (CS), a key enzyme in the *de novo* biosynthesis of ceramide and more complex sphingolipids (Wang *et al.*, 1991). Sphingolipids and sphingolipid metabolites are known to be important signaling molecules (Merrill *et al.*, 1997, 2001), and disruption of sphingolipid metabolism is a suspected cause of animal and plant diseases (Riley *et al.*, 2001). FBs are known causes of farm animal disease, including porcine pulmonary edema (PPE) and equine leukoencephalomalacia (ELEM) (WHO, 2000, and references therein).

In animals and plants, inhibition of CS results in an accumulation of free sphinganine (Sa), a toxic upstream precursor of ceramide (Merrill *et al.*, 1997). There are several ways in which cells can reduce intracellular Sa. One mechanism is to phosphorylate Sa (Sa kinase) to sphinganine 1-phosphate (Sa-1-P) and then hydrolyze Sa-1-P (Sa phosphate lyase) to phosphoethanolamine and a fatty aldehyde (Fig. 1). While the metabolism of sphingosine (So) to sphingosine 1-phosphate (So-1-P) has been well studied in cultured cells and some mammalian tissues, very little is known about the metabolism of accumulated So and Sa to their corresponding 1-phosphates in tissues from animals fed diets containing FB. Studies in pigs consuming FB diets found that serum concentrations of Sa and So-1-P and Sa-1-P were significantly increased (Piva *et al.*, 2005). A recent study in horses showed that Sa-1-P is also elevated in serum from horses dosed with fumonisin B_1 (FB_1) and that the elevation occurred at dosages that induced neurotoxicity (Constable *et al.*, 2005). These reports of FB-induced elevation in sphingoid base 1-phosphates are important for two reasons. First, Sa-1-P, like free Sa, is extremely low in pig and horse serum; however, the increase in the 1-phosphates in serum was greater than Sa. Thus, the elevation in Sa-1-P may prove to be a more reliable marker for FB exposure in pigs and horses. Second, So-1-P and Sa-1-P have been shown to be ligands for a family of extracellular G protein–coupled receptors in the cardiovascular tissue of animals known as S1P receptors (formerly endothelial differentiation gene receptors) (Bischoff *et al.*, 2000; Spiegel and Milstien, 2002). So-1-P, but not Sa-1-P, is also a suspected intracellular second messenger in pathways regulating calcium homeostasis (Spiegel and Milstien, 2002). Bischoff *et al.* (2000) found that sphingoid bases and sphingoid base metabolites affect the resistance of rat renal and mesenteric vessels

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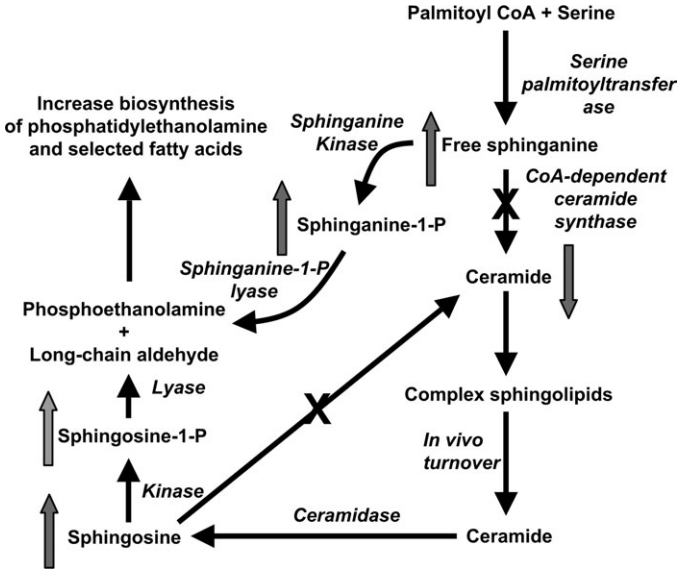

FIG. 1. *De novo* sphingolipid biosynthetic pathway and degradation pathway showing point of disruption by FB_1. Since Sa is an intermediate and not a product of *de novo* biosynthesis, its elimination is termed degradation. Gray arrows indicate the known effects on the metabolite pools *in vivo* as a result of disruption by FB_1.

in vitro and *in vivo* through a mechanism involving a G protein–coupled receptor. Thus, the marked elevation in both So-1-P and Sa-1-P could contribute to the vascular effects that are hypothesized to be the cause of FB-induced PPE (Hsiao *et al.*, 2005) and ELEM (Constable *et al.*, 2005) and possibly effects in other tissues. Recently, it was shown that So-1-P acting via the $S1P_3$ receptor induces pulmonary edema in mice by disrupting pulmonary epithelial tight junctions, and it was hypothesized that $S1P_3$ regulation of epithelial integrity could be involved in pathologies in other organ systems (Gon *et al.*, 2005). Thus, understanding the effect of FB on sphingoid base 1-phosphate levels in blood and target tissues is important for understanding the potential for physiological effects on target tissues.

In male Sprague-Dawley and Fischer 344N rats, the kidney is the most sensitive target for FB toxicity (Howard *et al.*, 2001; NTP, 2001; Voss *et al.*, 2001, and references therein). In feeding studies with pure FB_1, elevation of Sa in rat kidney parallels the extent and severity of nephrotoxicity (NTP, 2001; Riley *et al.*, 1994). The present study in male Sprague-Dawley rats fed diets containing FB was undertaken to investigate the early time- and dose-dependent changes in accumulation of free sphingoid bases and their 1-phosphates in serum, heart, liver, and kidney of animals fed diets containing FB. The amount of FB in liver and kidney was also measured and correlated with pathology and elevation in So, Sa, So-1-P, and Sa-1-P. The ultimate purpose is to establish in future studies the lowest dietary exposure and tissue concentration of FB that can elicit accumulation of sphingoid bases and their metabolites and toxicity in rat kidney which was the critical target organ used by the WHO/FAO Joint Expert Committee on Food Additives for calculating the current provisional maximum tolerable daily intake for FB (Bolger *et al.*, 2001). Determining the lowest effective dose will allow the design of *in vivo* mechanistic studies using combinations of inhibitors of sphingolipid metabolism with minimal risk of the confounding toxicity associated with concurrent use of multiple metabolic inhibitors.

MATERIALS AND METHODS

Diet preparation and feeding studies. Male Sprague-Dawley rats (3–4 weeks of age) were fed Teklad LM 485 (Teklad, Madison, WI) rodent chow *ad libitum* or test diets consisting of rodent chow containing *Fusarium verticillioides* maize culture material containing FB. Culture material of *F. verticillioides* strain MRC 826, an FB-producing isolate (WFO Marasas, Medical Research Council, Tygerburg, South Africa), was prepared as previously described (Voss *et al.*, 1996), freeze dried, ground, and mixed with control rodent chow (Teklad LM 485) using a Patterson Kelley V blender with intensifier bar to make the diets. The total FB ($FB_1 + FB_2 + FB_3$) based on liquid chromatography mass spectrometry (LCMS) (method described below) were 1.1, 13.5, and 88.6 ppm for control diet (CD = unamended rodent chow), low-dose (LD), and high-dose (HD) diets, respectively. The ratio of FB_1:FB_2:FB_3 in the diets was 1.00:0.45:0.10 (wt/wt/wt). There were 12 rats in each group (CD, LD, and HD), and three from each group were killed after 1, 3, 5, or 10 days of feeding. The animals were assigned to groups using a random block design by body weight. Mean body weights of the three groups at the beginning of the study were 142 ± 8.4 (SD), 141 ± 5.8, and 141 ± 5.9 for the CD, LD, and HD groups, respectively. Animals were individually housed in stainless steel wire mesh cages and, to avoid reversal of sphingolipid effects, not fasted before necropsy. The experiments conformed to the federal guidelines for the use and care of laboratory animals and were approved by the Russell Research Center Institutional Animal Care and Use Committee.

Observations, histopathology, and scoring. Animals were observed daily and their total feed consumption and body weights measured immediately before necropsy. The rats were anesthetized with isoflurane USP (Isoflo, Abbott Laboratories, North Chicago, IL), and blood was collected from the periorbital plexus for preparation of serum. The rats were then euthanized with carbon dioxide and the liver, kidney, and heart excised and weighed and specimens of each fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin for microscopic examination or, along with an aliquot of serum, frozen (−80°C) for sphingolipid analysis.

Liver, kidney, and heart specimens from each rat were microscopically examined, in random order and without knowledge of the animals' identity, for lesions consistent with those induced by FB (Howard *et al.*, 2001; NTP, 2001; Voss *et al.*, 2001). Liver and kidney (focusing on the tubules found in the outer medulla) lesions were assigned scores according to the following criteria. For liver, a score of 0 indicates that the tissue was "normal" in appearance. A score of 1 was assigned to those specimens showing a minimal effect, which was defined as a few widely distributed apoptotic hepatocytes scattered throughout an otherwise normal parenchyma. A liver score of 2 (mild effect) was given when there were obvious lesions characterized by more frequent apoptosis and changes in hepatocyte cell (anisocytosis and cytomegaly) and nucleus (anisokaryosis) size. Specimens showing qualitatively similar (as described for 2) but more extensive lesions were scored as 3, while a score of 4 was reserved for severely damaged livers characterized by a spectrum of findings including increased apoptosis and mitosis, oncotic necrosis, cytomegaly and anisocytosis, anisokaryosis, inflammation, fibrosis, bile duct or oval cell proliferation, or focus of cell alteration or hyperplasia. Because the findings in liver were subtle (with two exceptions, all scores were 0 or 1), the hepatic

effect of FB was further evaluated by counting the number of apoptotic hepatocytes in 15 random high-power (10× ocular, 40× objective lenses) fields (Sharma *et al.*, 2002).

Kidney specimens that were judged normal in appearance were assigned scores of 0. A score of 1 was given when a few apoptotic epithelial cells were observed in the tubules of the outer medulla of an otherwise normal tissue. A score of 2 indicated that obvious lesions consistent with FB exposure were present. The criteria for a score of 2 included apoptotic tubule epithelial cells scattered throughout the outer medulla, detachment and sloughing of the apoptotic cells, and occasional mitotic figures. Lesions exhibiting more extensive tubular epithelial apoptosis and sloughing along with increased cytoplasmic basophilia, anisocytosis, anisokaryosis, decreased height of the epithelium, and occasional focal tubular hyperplasia (indicative of regeneration) were assigned a score of 3. A score of 4 was indicative of severe lesions (not seen in this study). Criteria for a score of 4 included extension of the lesions into the cortex with overt tubular (oncotic) necrosis and (focally) complete loss of tubule epithelium due to sloughing of apoptotic cells, tubular atrophy, interstitial inflammation, and fibrosis.

Extraction of FBs, free sphingoid bases, and sphingoid base 1-phosphates. The FBs were extracted from diets as described by Rice *et al.* (1995). Samples of liver and kidney homogenates (20–40 mg total tissue) were also extracted and analyzed for FB except that the extraction solvent was acetonitrile:10% formic acid (1:1, vol/vol), and extraction was done in polypropylene tubes with sonication for 10 min at 37°C. The use of formic acid has been shown to extract FB from soils in which FB cannot be extracted with either water or acetonitrile:water (1:1, vol/vol) (Williams *et al.*, 2003). Extracts were diluted with water and passed through conditioned C_{18} solid-phase Sep-Pak cartridges (Waters, Milford, MA). Bound FBs were eluted and analyzed by LCMS (described below).

For sphingolipids the extraction method was a modification of Sullard and Merrill (2001). Briefly, kidney, liver, and heart samples were homogenized in cold phosphate buffer and then aliquots transferred to glass tubes. A total of 0.6 ml methanol/100 µl and 0.3 ml chloroform/100 µl plus 10 µl internal standard (10 ng/µl C_{17} So-1-P and 50 ng/µl C_{20} Sa, Avanti Polar Lipids, Inc., Alabaster, AL) was added to each sample. Samples were sonicated for 1 min at room temperature, capped tightly, and incubated overnight at 48°C in a heating block. After cooling, 75 µl of 1M methanolic KOH was added, and the samples were sonicated and incubated for 2 h at 37°C. The samples were then centrifuged at 20,000 rpm for 10 min, and the supernatants were transferred to glass tubes. The samples were neutralized with 1 *N* HCl and then evaporated to dryness in a vacuum centrifuge without heat and stored under N_2 at − 20°C. The dried samples were reconstituted in acetonitrile:water:formic acid (49.5:49.5:1, vol/vol) containing 5mM ammonium formate and clarified by filter centrifugation using a 0.45-µm Nylon Microspin filter (Lida Manufacturing Corp., Kenosha, WI). The solubilized samples were then analyzed by LCMS.

LCMS method. FBs were separated on a Thermal Separations high-performance liquid chromatograph (Riviera Beach, FL) consisting of a model P2000 solvent delivery system and an AS3000 autosampler. Separations were done using an Intersil 5 µ octadecyl silane (ODS)-3 column (150 × 3 mm, Metachem Technologies, Inc., Torrance, CA). The flow was 0.2 ml/min, and the mobile phase was a 28-min programmed gradient starting at 30% of 97% acetonitrile:2% water:1% formic acid (solvent A) and 70% of 2% acetonitrile:97% water:1% formic acid (solvent B), and after 15 min the proportions of A and B were 60 and 40%, respectively, and at 20 min the proportions of A and B were 90 and 10%, respectively followed by a 8-min gradient returning to 30% A and 70% B. The total run time was 28 min, and there was a 5-min equilibration between each injection. The column effluent was directly coupled to a ThermoFinnigan LCQ Duo ion trap mass spectrometer (MS) (Woodstock, GA). The MS was operated in the electrospray ionization (ESI) positive-ion mode with an inlet capillary temperature of 190°C, and the sheath gas was nitrogen. For MS/MS of FB_1, FB_2, and FB_3, the collision energy was 32%, and mass fragments were scanned from 195 to 800 *m/z* and compared to authentic standards.

Sphingoid bases and sphingoid base 1-phosphates were chromatographically separated (Fig. 2) on the same LCMS system as FB; however, the gradient started at 50% solvent A and at 15 min it was 70% solvent A and at 20 min it was 100% solvent A which was held until 25 min at which time the column was reequilibrated with 50% A for 15 min before the next injection (10–30 µl). The total run time was 40 min. The MS was operated in the ESI positive-ion mode with an inlet capillary temperature of 170°C, and the sheath gas was nitrogen. For MS/MS the collision energy was 30%. MS/MS mass fragments were

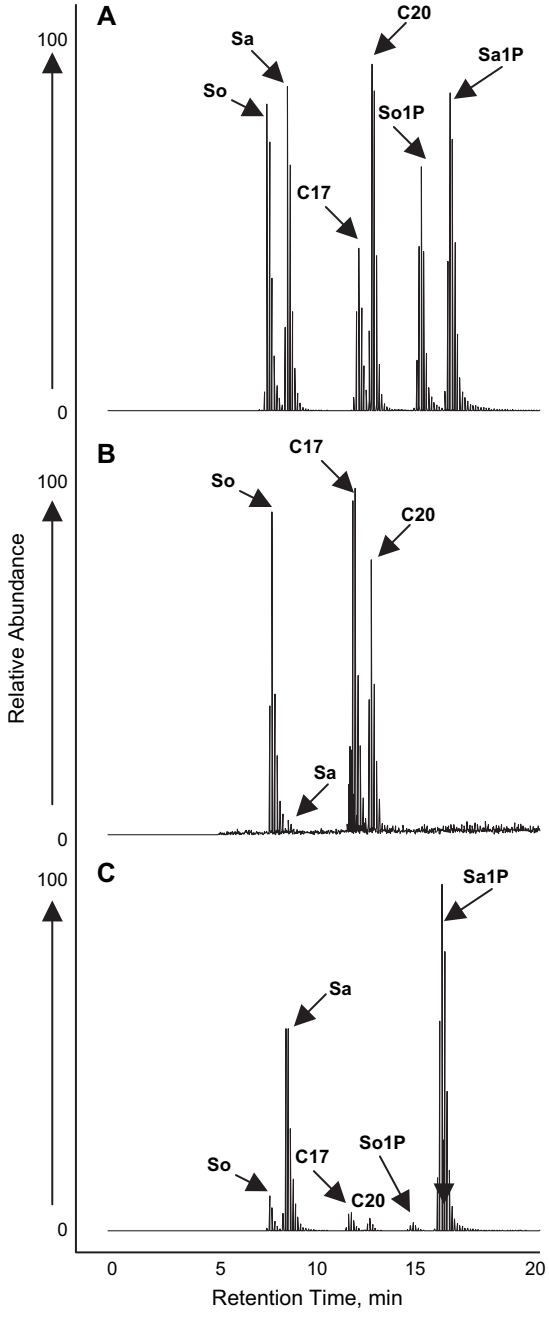

FIG. 2. Chromatogram showing the total ion current for a standard (A) containing So, Sa, C_{20} Sa (C20), C_{17} So-1-phosphate (C17), So-1-P, and Sa-1-P (total injection = 2–4 ng each compound), a control kidney extract (B) and extract of a HD kidney (C).

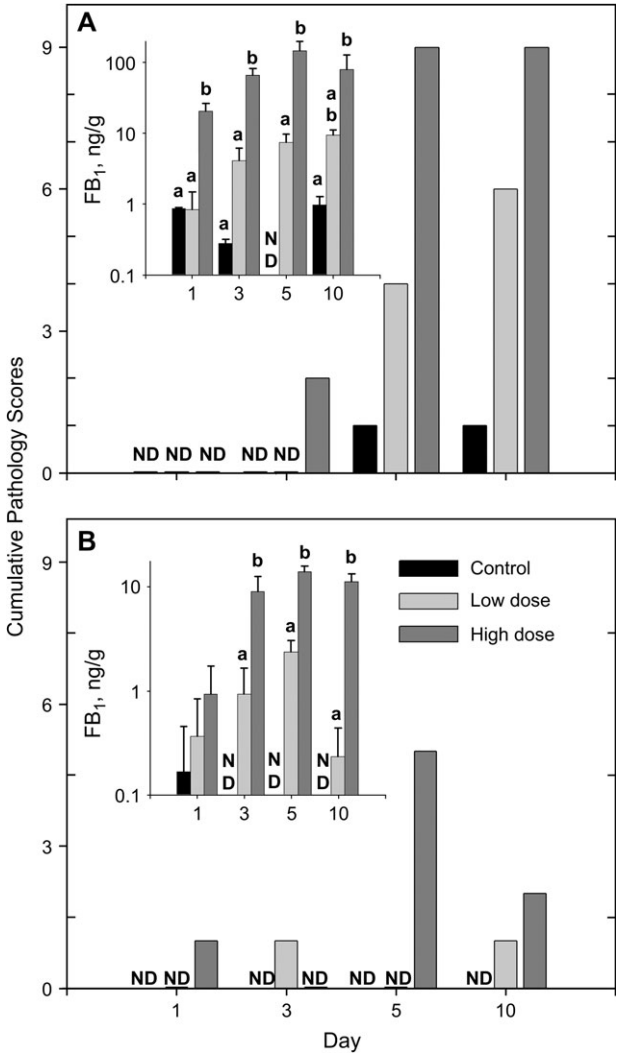

FIG. 3. The kidney (A) and liver (B) cumulative pathology scores (sum of scores for all rats) on days 1, 3, 5, and 10 for rats fed diets containing *Fusarium verticillioides* culture material that contained 1.1, 13.5, and 88.6 µg/g of total FB ($FB_1 + B_2 + B_3$) in the control, LD, and HD diet treatment groups, respectively. Inset is the FB_1 level in kidney and liver at each sample time. ND indicates that no histological abnormalities were seen or the FB_1 content was below the detection limits of the method. For the FB_1 levels (inset), groups fed control, LD, or HD diets (for each time point) with differing superscripts are significantly different ($p < 0.05$), and if there were no significant differences among groups, then there are no letters above the bars for the group.

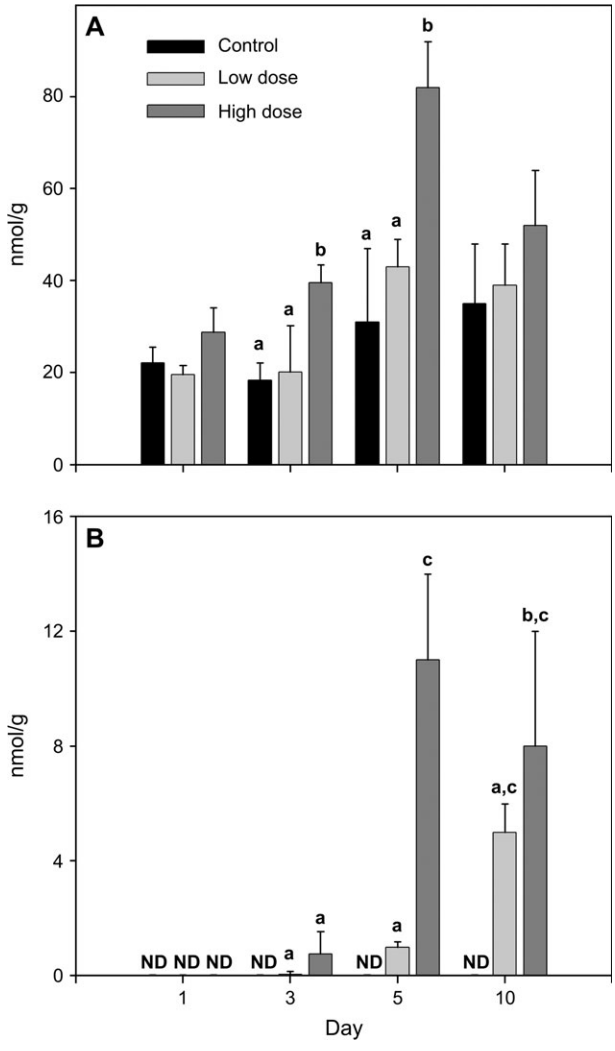

FIG. 4. Free So (A) and So-1-P (B) in kidney of rats treated as described in Figure 3. Values are means ± SDs ($n = 3$). Groups fed control, LD, or HD diets (for each time point) with differing superscripts are significantly different ($p < 0.05$), and if there were no significant differences among groups, then there are no letters above the bars for the group. ND indicates not detected.

scanned from 195 to 400 *m/z* and compared to authentic standards. Some samples were also analyzed using data-dependent scanning of the two most intense ions (150–600 *m/z*).

Statistical analysis. Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). Where many groups were compared one-way ANOVA was used, followed by *post hoc* multiple comparisons. The Pearson product moment correlation was used to measure the strength of the association between pairs of variables. Where only two groups were compared, the Student *t* test was used for parametric data, and the Mann-Whitney rank sum test was used for nonparametric data. All data were expressed as mean ± SD, and differences among means were considered significant if the probability (p) was ≤ 0.05.

RESULTS

Clinical observation of the animals revealed no evidence of a toxic effect (data not shown). There were no significant differences in body weight at any time. Nonetheless, the mean body weight of the HD group (177 ± 7.4 g) was 10–11% less than that of the LD (196 ± 6.7 g) and control (199 ± 14.0 g) groups on day 10. Likewise, feed consumption was 20% less in the HD group and 6% less in the LD group compared to the control group on day 10 (not significant).

Absolute kidney weight of the LD group (1.56 ± 0.07 g) was significantly less than that of the control group (1.68 ± 0.03 g) on day 10. Kidney weight of the HD group (1.40 ± 0.04 g) was in turn significantly less than both the control and LD groups. There were no significant differences in relative (% body

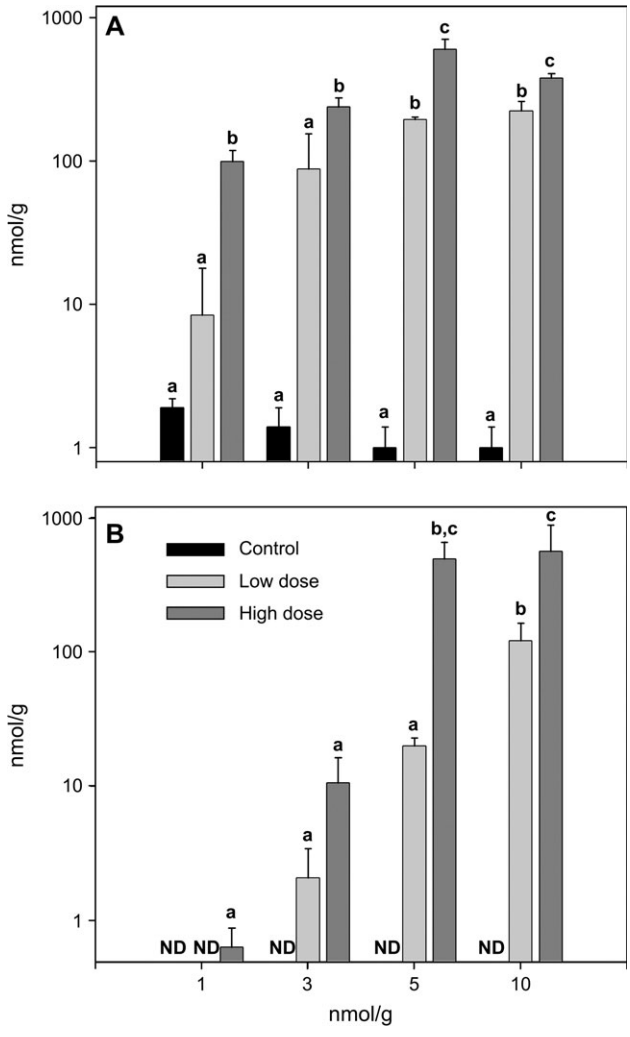

FIG. 5. Free Sa (A) and Sa-1-P (B) in kidney of rats treated as described in Figure 3. Values are means ± SDs ($n = 3$). The data are presented as the $\log_{10}$ values because the magnitude of the differences between values at the early time points (days 1 and 3) are not easily seen in a linear presentation of the data. Groups fed control, LD, or HD diets (for each time point) with differing superscripts are significantly different ($p < 0.05$), and if there were no significant differences among groups, then there are no letters above the bars for the group. ND indicates not detected. The limit of detection for C_{17} So-1-P is 0.5 nmol/g.

weight) kidney weights: relative kidney weights of the LD (0.79 ± 0.03%) and HD (0.79 ± 0.02%) groups were about 6% less than those of the controls (0.85 ± 0.05%) on day 10. Absolute and relative liver weights of the HD group were significantly less than those of the controls on day 5 and those of both the control and LD groups on day 10. Values on day 5 were as follows: controls = 7.5 ± 0.44 g (relative weight = 3.76 ± 0.22%), LD = 6.9 ± 0.58 g (3.52 ± 0.30%), and HD = 5.91 ± 0.03 g (3.34 ± 0.02%). Results from animals examined on day 10 were as follows: controls = 9.28 ± 0.55 g (4.66 ± 0.07%), LD = 8.76 ± 0.17 g (4.47 ± 0.15%), and HD = 6.94 ± 0.46 g (3.91 ± 0.09%).

Histological changes consistent with those known to be caused by pure FB_1 (Howard *et al.*, 2001; NTP, 2001; Voss *et al.*, 2001) and detailed in the description of scoring criteria in the ''Materials and Methods'' section, were observed in both kidney and liver in the LD and HD groups. Microscopic examination revealed no evidence of any heart lesions such as those that have been reported in animals fed diets containing FB from *Fusarium proliferatum* culture material (Piva *et al.*, 2005).

Briefly, pathological effects in kidney were both time and dose dependent (Fig. 3A) and involved the tubules of the outer medulla. The kidneys of the control group were unremarkable. However, one control kidney was given a score of 1 on each of days 5 and 10. This score was based on the appearance of one or more apoptotic cells, which are a sporadic background finding in untreated rats, in the outer medulla (the target zone of FB in male rat kidneys) in an otherwise normal-appearing parenchyma. Evidence for an FB effect was not found in the LD group on days 1 or 3, but all LD rats killed on days 5 or 10 exhibited lesions. These lesions were minimal (two rats) to mild (one rat) in severity on day 5 and minimal to moderate on day 10. In the HD group, the FB effect occurred earlier than in the LD group and was more severe, as all lesions were judged to be moderate at days 5 and 10.

Liver lesions were subtle, and therefore, the assignment of scores for ''FB-like'' lesions depended almost exclusively on the presence of apoptotic hepatocytes and, for the few more affected livers, variability in cell and nuclear size. Scores for individual animals did not exceed 1 (minimal effect) except for the HD group on days 5 and 10 (Fig. 3B). The greatest effect was seen on day 5. The three HD rats killed that day had apoptosis counts of 8, 18, and 34 per 15 high-power fields; the respective scores for these animals were 1, 2 (mild effect), and 2. Fewer apoptotic hepatocytes (counts were 0, 2, and 3) and no scores above 1 were found in the HD group on day 10. The diets had no obvious effect on mitosis counts. Most (28 of the 36) animals had total mitotic figure counts of ≤ 5. A few had higher numbers; the highest counts for individual rats in the CD, LD, and HD groups were 17 (day 10), 16 (day 10), and 10 (days 1 and 5).

FB_1 was detected in both kidney and liver of LD and HD animals at all sample times. FB_1 was also detected in kidney and liver of several rats fed the CDs. Surprisingly, FB_2 and FB_3 were only detected in liver and kidney in the animals fed the HD diets, and the mean levels in tissues were significantly less than those in the diets ($p < 0.001$); FB_2 concentration in the diets comprised 29 ± 4.3% ($n = 9$) of the total FB, whereas the FB_2 in the liver and kidney was only 4.0 ± 3.7% ($n = 13$) of the total FB. Nonetheless, there was a clear dose- and time-dependent increase in FB_1 in kidney and liver, and the concentrations of FB_1 in kidney were much greater than those in liver (Figs. 3A and 3B, inset); e.g., the mean concentration of FB_1 in kidney and liver on day 5 in HD animals ($n = 3$) was 145 ± 50 ng/g and 14 ± 2 ng/g, respectively, and the difference was

TABLE 1
Pearson Product Moment Correlation for Kidney Free So, free Sa, So-1-P, Sa-1-P, FB_1, and Pathology Scores (Path) for Individual Rats ($n = 36$) at All Dose Levels and Times[a]

	Sa	So-1-P	Sa-1-P	FB_1	Path
So					
Correlation	0.89	0.78	0.70	0.81	0.69
p	6.0×10^{-13}	2.9×10^{-8}	1.7×10^{-6}	2.16×10^{-9}	3.8×10^{-6}
Sa					
Correlation		0.86	0.81	0.92	0.78
p		1.04×10^{-11}	2.6×10^{-8}	5.4×10^{-15}	2.9×10^{-8}
So-1-P					
Correlation			0.94	0.84	0.78
p			1.1×10^{-17}	1.7×10^{-10}	2.7×10^{-8}
Sa-1-P					
Correlation				0.83	0.76
p				4.5×10^{-10}	7.1×10^{-8}
FB_1					
Correlation					0.68
p					4.4×10^{-6}

[a]The pair of variables with positive correlation coefficients and *p* values below 0.050 (5×10^{-2}) tend to increase together. The exact *p* values are presented in exponential format.

significant ($p < 0.01$). The lowest mean FB_1 concentration in kidney and liver that was associated with a cumulative pathology score greater than 2 was 7 ng/g (LD day 5) and 14 ng/g (HD day 5), respectively (Figs. 3A and 3B).

Changes in free sphingoid bases and sphingoid base 1-phosphates were detected prior to histological changes in kidney (Figs. 4 and 5). Small but significant increases in So were only apparent in the HD group on days 3 and 5 (Fig. 4A). So-1-P was not detected in any treatment group on day 1 and was not detected in control kidney on any day (Fig. 4B). So-1-P was first detected in kidney on day 3 in both the LD and HD groups and was maximal in the HD group on day 5 and the LD group on day 10 (Fig. 4B). Sa and Sa-1-P were first elevated at day 1 in the HD group, and in the LD group there was a marked increase (40-fold) in mean Sa and a significant increase in Sa-1-P on day 3 (Figs. 5A and 5B). Both Sa and Sa-1-P were maximally elevated at day 5 to similar levels. Sa-1-P was not detected in the LD kidney on day 1 or in control kidney on any day (Fig. 5B). The maximal amounts of Sa-1-P were 50-fold greater than the levels of So-1-P (Figs. 2C, 4B, and 5B). Overall, in kidney there was a highly significant statistical correlation between the elevation in free sphingoid bases, sphingoid base 1-phosphates, FB_1 tissue content, and the pathology scores (Table 1). The best correlation was between Sa and FB_1, and the correlation between Sa and sphingoid base 1-phosphates and the pathology scores were all better than that between FB_1 and the pathology scores (Table 1).

In liver there was a small but significant increase in So only in the HD group on day 10 (Fig. 6A). Increases in free Sa were detected prior to histological changes in liver. Sa was slightly but significantly elevated in the HD group on day 1 and maximally elevated on day 5 (Fig. 6B). There was no So-1-P detected in liver from control or FB-fed animals, and only one liver from the HD group had any detectable Sa-1-P (data not shown); the limit of detection for C_{17} So-1-P was 0.5 nmol/g. Nonetheless, overall in liver there was a highly significant statistical correlation between the elevation in Sa, FB_1 tissue content, and the pathology scores (Table 2). As in kidney, the correlation between Sa and the pathology scores was better than the correlation between FB and the pathology scores.

The amount of FB in the liver and kidney that is necessary to elicit a minimum/mild morphologic change was similar, but the amount of sphingoid bases and sphingoid base 1-phosphates accumulated was much greater in kidney. In liver and kidney of FB-exposed rats that exhibited minimum (score = 1) or mild (score = 2) histopathological effects, there was no significant difference in the mean calculated FB_1 molar concentration, whereas, the molar concentration of total Sa (Sa plus Sa-1-P) in kidney was 14 times greater than that in liver (Fig. 7).

In the serum there was no effect on So (data not shown) or So-1-P (data not shown), whereas Sa was significantly elevated on days 5 and 10 (Fig. 8A) in the HD group, and Sa-1-P was significantly elevated on days 3, 5, and 10 (Fig. 8B). The maximal increase in both Sa and Sa-1-P was on day 5. FB_1 was also detected in the serum of all HD animals (Fig. 8B, inset). The highest level detected was 1 ng/ml of serum, much less than the highest levels seen in liver and kidney. FB_2 was also detected in some serum samples but as seen in liver and kidney, the relative amount was much less than expected based on the FB_2 level in the diets; serum $FB_2 = 12.7 \pm 7.2\%$ ($n = 6$) of the total FB.

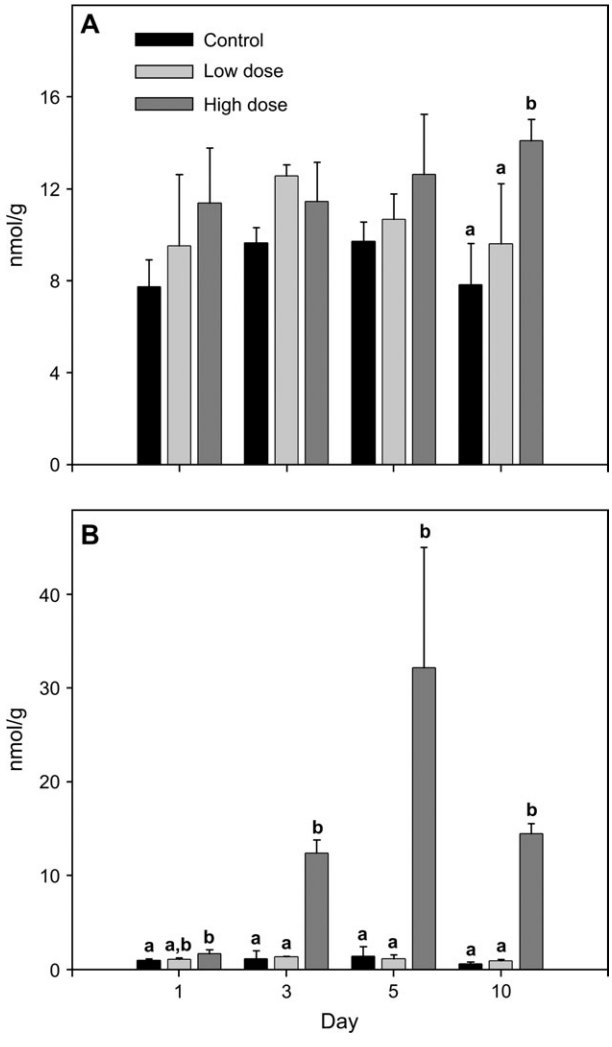

FIG. 6. Free So (A) and free Sa (B) in liver of rats treated as described in Figure 3. Values are means ± SDs ($n = 3$). Groups fed control, LD, or HD diets (for each time point) with differing superscripts are significantly different ($p < 0.05$), and if there were no significant differences among groups, then there are no letters above the bars for the group.

In the heart, significant increases in both So and Sa were only seen in the HD group on day 5 (Figs. 9A and 9B). FB_1, So-1-P, and Sa-1-P were not detected in heart tissue at any dose or time.

DISCUSSION

FB is a potent inhibitor of CS. The concentration of FB_1, sphingoid bases, and sphingoid base 1-phosphates in rat heart, liver, and kidney (heart <<< liver << kidney) are correlated with the susceptibility to morphologic injury. In this study, the increased accumulation of FB_1 in rat kidney provides a plausible explanation for the marked sensitivity of male Sprague-Dawley and Fischer 344N rats to FB-induced renal toxicity and disruption of sphingolipid metabolism.

TABLE 2
Pearson Product Moment Correlation for Liver Free So, Free Sa, FB_1, and Pathology Scores (Path) for Individual Rats ($n = 36$) at All Dose Levels and Times[a]

	Sa	FB_1	Path
So			
Correlation	0.46	0.51	0.39
p	5.2×10^{-3}	1.5×10^{-3}	2.0×10^{-2}
Sa			
Correlation		0.84	0.76
p		9.3×10^{-11}	1.0×10^{-7}
FB_1			
Correlation			0.65
p			1.9×10^{-5}

[a]The pair of variables with positive correlation coefficients and p values below 0.050 (5×10^{-2}) tend to increase together. The exact p values are presented in exponential format.

In male Sprague-Dawley and Fischer 344N rats (NTP, 2001; Voss *et al.*, 2001, and references therein), the accumulation of sphingoid bases and toxicity in kidney are much greater than that in liver at the same dose; a result consistent with preferential accumulation of FB in rat kidney. In all rat strains studied (Sprague-Dawley, BD IX, and Wister), the small amount of FB that is absorbed is found primarily in liver and kidney (Martinez-Larranaga *et al.*, 1999; Norred *et al.*, 1993; Shephard *et al.*, 1992). In BD IX rats more ^{14}C-FB_1 was recovered from liver than from kidney (Shephard *et al.*, 1992); liver is the primary target organ in male BD IX rats (Gelderblom *et al.*, 1991). In male Sprague-Dawley rats the specific activity of ^{14}C-FB_1 in liver and kidney after three daily doses remained elevated at similar levels for at least 96 h after the last dose (Norred *et al.*, 1993). However, in male Sprague-Dawley rats and Fischer 344N rats, the kidney is more sensitive to both FB-induced histopathological effects and disruption of sphingolipid metabolism compared to liver (NTP, 2001; Voss *et al.*, 2001). In male Wistar rats following a single oral dose of FB_1, the kidney contained almost 15 times more FB_1 than the liver (Martinez-Larranaga *et al.*, 1999), a result that is consistent with this study; Martinez-Larranaga *et al.* (1999) did not microscopically examine the tissues. It is possible that the studies using ^{14}C-FB_1 (which required sample combustion, liquid scintillation counting, and equated recovered ^{14}C-CO_2 to FB) underestimated the FB_1 in the kidney.

Other factors that could contribute to the increased accumulation of free sphingoid bases and sphingoid base 1-phosphates in kidney compared to liver include: (1) CS in rat kidney *in vivo* could be more sensitive to FB inhibition; (2) metabolism or alternate pathways for elimination of sphingoid bases or their 1-phosphates could be more active in liver; and (3) kidney could accumulate sphingoid bases and their 1-phosphates from the blood. The evidence supporting each possibility will be considered.

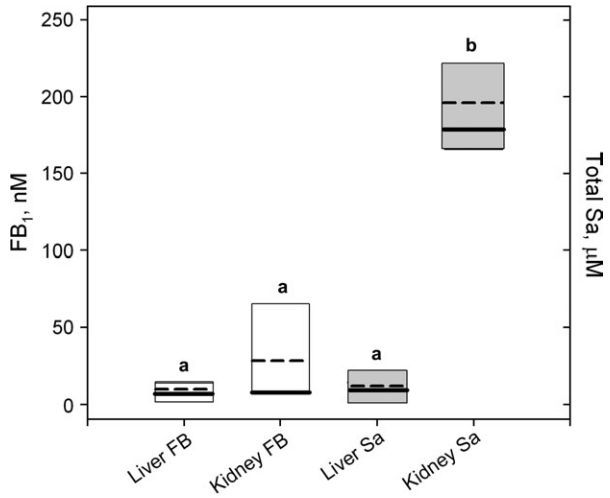

FIG. 7. A box plot showing the calculated molar concentration (assumed water content was 80% of wet weight) of FB_1 and total Sa (Total Sa = free Sa plus Sa-1-P) concentration in liver ($n = 8$) and kidney ($n = 7$) of all FB-fed rats with pathology scores of ''minimal'' (1) or ''mild'' (2). Only values for tissues scored minimum or mild were used in order to compare the tissue levels of FB_1 necessary to elicit a minimum/mild morphologic change and the respective elevation in total Sa. The lower and upper boundary of each box represents the 25th and 75th percentile, respectively. The dotted line is the mean and the heavy solid line in each box the median value for each group. Differing superscripts indicate that the differences for FB_1 or Total Sa are significantly different ($p \leq 0.05$).

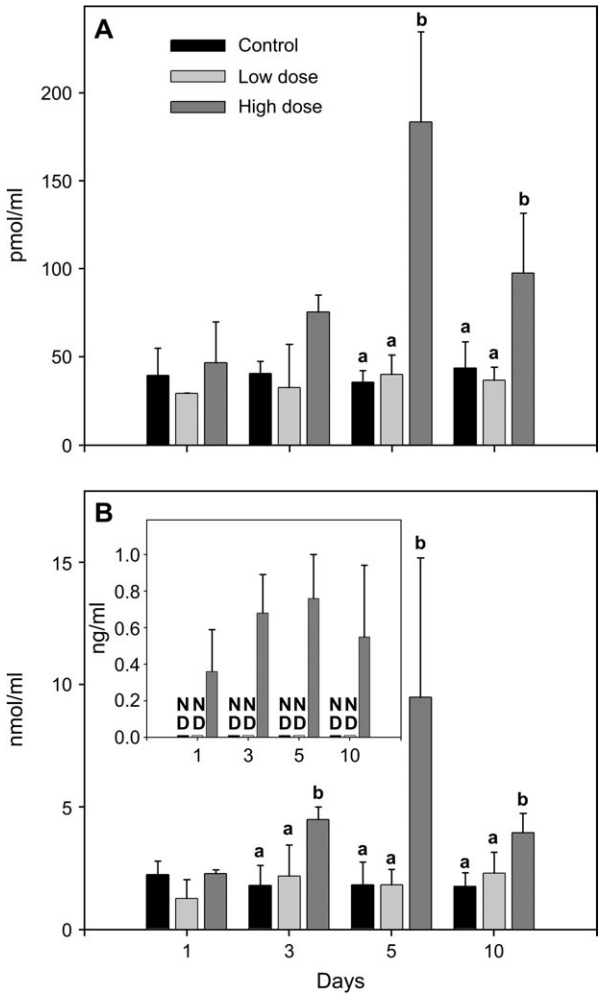

FIG. 8. Free Sa (A) and Sa-1-P (B) in serum of rats treated as described in Figure 3. Values are means ± SDs ($n = 3$). Groups fed control, LD, or HD diets (for each time point) with differing superscripts are significantly different ($p < 0.05$), and if there were no significant differences among groups, then there are no letters above the bars for the group. The levels (means ± SDs, $n = 3$) of FB_1 detected in serum are inset in (B). ND = none detected.

FB_1 appears to be an equally potent inhibitor of CS in liver and kidney *in vivo*. It is thus highly unlikely that liver in male Sprague-Dawley rats accumulates less free sphingoid bases and their 1-phosphates because it contains a CS resistant to FB inhibition. In tomato plants, resistance to FB toxicity is attributed to the *Asc-1* gene, which is now known to be homologous to yeast longevity assurance genes (Brandwagt *et al.*, 2000), which confer resistance to FB inhibition of the CoA-dependent CS in yeast (Lynch and Dunn, 2004). A mammalian homolog of *Asc-1* has been identified and when overexpressed in human embryonic kidney cells causes increased biosynthesis of dihydroceramide and is not blocked by FB (Riebeling *et al.*, 2003). In rat primary hepatocytes and liver microsomes, the IC_{50} for FB inhibition of CS is 0.1µM (Wang *et al.*, 1991). In this study, the calculated molar concentration of FB_1 in liver (HD day 3) and kidney (LD day 5) first associated with significant elevation in total Sa (Sa plus Sa-1-P) was 0.01 ± 0.004µM ($n = 3$) and 0.008 ± 0.002µM, respectively, indicating that *in vivo* FB_1 is an equally potent inhibitor of CS in liver and kidney. This conclusion is also supported by an earlier study (Riley *et al.*, 1994) showing that the dose-dependent, FB_1-induced decrease in complex sphingolipids was similar in both liver and kidney of male and female Sprague-Dawley rats even though free Sa was much greater in kidney.

The most likely explanation for the accumulation of Sa in rat kidney is the inability to degrade Sa-1-P at a rate sufficient to keep up with its biosynthesis. Earlier studies utilizing the increase of Sa as a marker of FB exposure have underestimated the disruption of sphingolipid metabolism since they have failed to take into account the Sa that has been metabolized. In LD rats, the rates of Sa and Sa-1-P accumulation in kidney, but not liver, exceeded the capacity of the processes responsible for their elimination. In cultured pig renal epithelial cells and mouse kidney, the marked elevation in Sa persists for several days after exposure to FB_1 ceases (Enongene *et al.*, 2002), suggesting that the elimination pathway is incapable of effectively metabolizing the accumulated Sa. In the cultured renal cells exposed to FB_1, if serine palmitoyltransferase is inhibited using myriocin, the Sa concentration returns to control levels within a few hours (Enongene *et al.*, 2002). This suggests that, in the presence of FB, the kinase is much less active than the serine palmitoyltransferase. This could also be the case

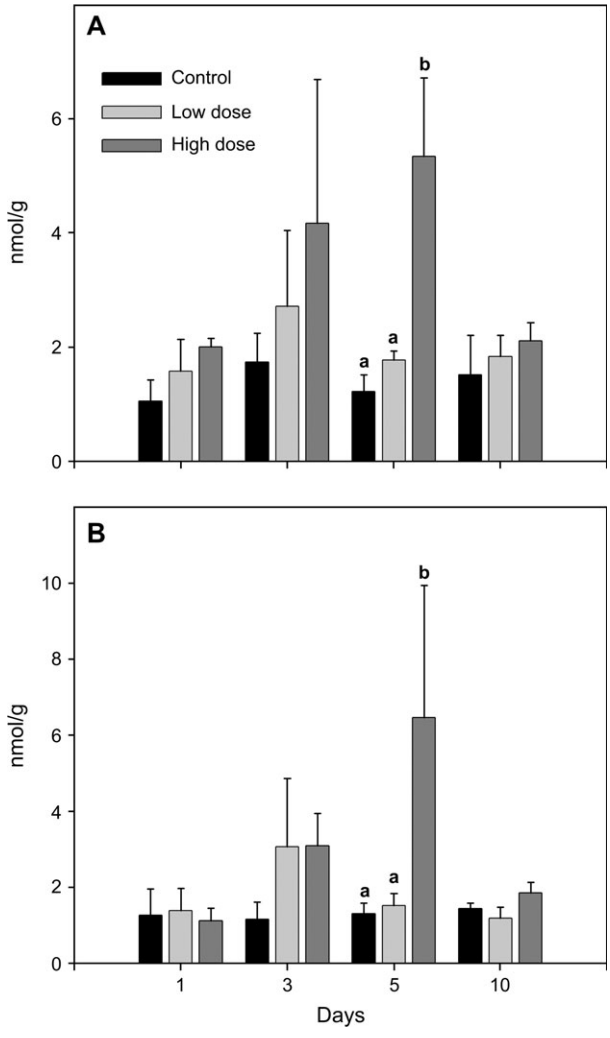

FIG. 9. Free So (A) and free Sa (B) in heart of rats treated as described in Figure 3. Values are means ± SDs ($n = 3$). Groups fed control, LD, or HD diets (for each time point) with differing superscripts are significantly different ($p < 0.05$), and if there were no significant differences among groups, then there are no letters above the bars for the group.

in vivo in rat kidney as evidenced by the marked accumulation of Sa, which precedes the accumulation of Sa-1-P. The accumulation of Sa-1-P indicates that the Sa phosphate lyase and/or phosphohydrolase are incapable of degrading the Sa-1-P as fast as it is made. Conversely, an explanation for the low levels of Sa in rat liver is that the liver eliminates Sa more effectively than kidney via Sa kinase and Sa-1-phosphate lyase or phosphohydrolase. This hypothesis is supported by the fact that the total Sa (Sa plus Sa-1-P) in kidney was much greater than that in the liver at similar tissues concentrations of FB_1 (Fig. 7).

A third possibility is that rat kidney may be able to take up Sa and/or Sa-1-P eliminated into the blood from other tissues. Norred *et al.* (1996) found that the level of Sa in kidney slices exposed to FB is actually less than that in the liver slices, suggesting that the source of the high level of accumulated Sa in rat kidney *in vivo* is not from *de novo* biosynthesis in the kidney. This could explain why the FB-induced elevation of Sa-1-P in serum (Fig. 8B) was much less than the FB-induced increase seen in pig serum (Piva *et al.*, 2005) and horse serum (Constable *et al.*, 2005), where kidney is not a target organ. The pattern of the Sa and Sa-1-P response in serum (Fig. 8) more closely reflected the response in liver (Fig. 6B) than the kidney and also paralleled the liver pathology (Fig. 3B), suggesting that the source of Sa in the serum was the liver.

The role of accumulated Sa-1-P in the pathological effects of FB in rat kidney is unknown. So-1-P, but not Sa-1-P, has been hypothesized to be an intracellular second messenger in pathways regulating calcium homeostasis and in activation of pathways involved in promoting cell survival (Hla, 2003; Spiegel and Milstien, 2002). However, the evidence for intracellular signaling indicates that Sa-1-P does not have any cytoprotective effect in the cell lines studied thus far (Van Brocklyn *et al.*, 1998). There is currently no data available as to the cytotoxicity of Sa-1-P or the effects of sustained elevation of sphingoid base 1-phosphates, in animal tissues; however, accumulation of phosphorylated long-chain bases in yeast causes growth inhibition (Kim *et al.*, 2000).

In addition to their possible role as intracellular second messengers, in mammals and yeast, So-1-P and Sa-1-P are also known to act as ligands for a family of extracellular G protein–coupled receptors known as S1P receptors (Spiegel and Milstien, 2002). There is currently no evidence for S1P receptors in tubular or glomerular cells of the rat kidney; however, if they are present, then the persistent elevation in sphingoid base 1-phosphates would have an adverse effect on their normal physiological function. Bischoff *et al.* (2000) demonstrated that So-1-P reduced renal blood flow in rats. This effect was abolished by pretreating the animals with pertussis toxin, suggesting that the vasoconstrictive effects of So-1-P were mediated by a mechanism dependent on $G_{(i)}$-type G proteins. Alternatively, elevated Sa-1-P in serum or tissues could disrupt S1P receptor signaling in the renal vasculature or epithelial cells by altering tight junctional integrity (Gon *et al.*, 2005).

In conclusion, in male rats consuming diets containing FB_1, FB_2, and FB_3, there is a time- and dose-dependent increase in Sa in both liver and kidney that is closely correlated with the tissue concentration of FB_1 and pathology. However, the Sa alone greatly underestimated the degree of disruption of sphingolipid metabolism since accumulated Sa and So can be quickly metabolized to Sa-1-P and So-1-P as evidenced by large increases in these metabolites in kidney but not liver. The liver and kidney tissue concentration of FB_1 that first elicits an increase in Sa is similar in both liver and kidney; however, kidney accumulates significantly more FB_1 and total Sa (Sa plus Sa-1-P) compared to liver. Thus, the relative sensitivity of male Sprague-Dawley rat kidney and liver is most likely a consequence of differences in the mechanisms responsible for both FB_1 uptake and Sa metabolism.

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